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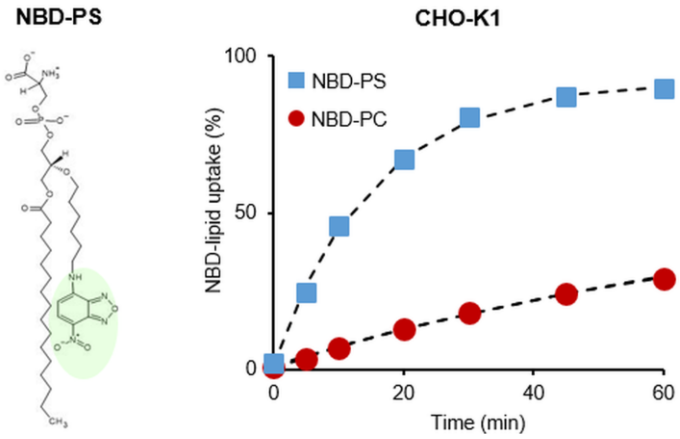
NBD-lipid Uptake Assay for Mammalian Cell Lines

Sara Abad Herrera[1], Marta Grifell-Junyent[1, 2, $] and Thomas Günther Pomorski[1, 2, *]

[1]Department of Molecular Biochemistry, Faculty of Chemistry and Biochemistry, Ruhr University Bochum, Bochum, Germany
[2]Department of Plant and Environmental Sciences, University of Copenhagen, Frederiksberg, Denmark
[$]Present address: Senolytic Therapeutics, Parc Científic de Barcelona, Barcelona, Spain
*For correspondence: Thomas.Guenther-Pomorski@ruhr-uni-bochum.de

[Abstract] All eukaryotic cells are equipped with transmembrane lipid transporters, which are key players in membrane lipid asymmetry, vesicular trafficking, and membrane fusion. The link between mutations in these transporters and disease in humans highlights their essential role in cell homeostasis. Yet, many key features of their activities, their substrate specificity, and their regulation remain to be elucidated. Here, we describe an optimized quantitative flow cytometry-based lipid uptake assay utilizing nitrobenzoxadiazolyl (NBD) fluorescent lipids to study lipid internalization in mammalian cell lines, which allows characterizing lipid transporter activities at the plasma membrane. This approach allows for a rapid analysis of large cell populations, thereby greatly reducing sampling variability. The protocol can be applied to study a wide range of mammalian cell lines, to test the impact of gene knockouts on lipid internalization at the plasma membrane, and to uncover the dynamics of lipid transport at the plasma membrane.

Graphic abstract:



Internalization of NBD-labeled lipids from the plasma membrane of CHO-K1 cells.

Keywords: Flow cytometry, Lipid transport, Mammalian cells, NBD-lipid, Plasma membrane

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[Background] A remarkable feature of many biological membranes is that their phospholipids are asymmetrically distributed across the lipid bilayer, a phenomenon known as transbilayer lipid asymmetry. This lipid asymmetry is essential for several vital cellular functions, including regulation of membrane protein activity, signaling, and vesicle formation in the secretory and endocytic pathways. Thus, establishing and regulating lipid asymmetry is crucial for cells, and a number of membrane proteins have evolved to fulfill the function of cross-bilayer lipid transporters. These transporters include ATP-dependent flippases and floppases—which catalyze the inward movement of lipids from the extracellular/luminal leaflet to the cytoplasmic leaflet, and the outward movement of lipids from the intracellular leaflet to the extracellular/ luminal leaflet, respectively, and ATP-independent scramblases (Holthuis and Levine, 2005; Contreras *et al.*, 2010). Despite their fundamental cellular importance, key aspects of how these lipid transporters operate await elucidation.

A subgroup of P-type ATPases, the P4-ATPases, emerged as a major group of lipid flippases that form heterodimeric complexes with members of the Cdc50 (cell division control 50) protein family (Lopez-Marques *et al.*, 2014). Mutations in these transporters generate impairments in physiological processes and, in humans they have been linked to diseases such as intrahepatic cholestasis and cerebellar ataxia (van der Mark *et al.*, 2013). While initially characterized as aminophospholipid flippases, recent studies of individual family members from yeast, parasites such as *Leishmania*, plants, and mammalian cells show that P4-ATPases differ in their substrate specificities and mediate transport of a broader range of lipid substrates, including lysophospholipids, synthetic alkylphospholipids, and glycolipids (Roland *et al.*, 2019; Shin and Takatsu, 2019).

Characterization of phospholipid movement at a quantitative level in the plasma membrane of eukaryotic cells is frequently based on fluorescent lipids with a covalently linked 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) group in the *sn-2* position. These lipid analogs have a NBD group attached to a short fatty acid chain (C6) and maintain most of the properties of endogenous phospholipids, except that they are more water-soluble, which facilitates incorporation from the medium into the outer monolayer of the plasma membrane. Transport of these probes is usually monitored by extracting the residual fraction of analogs not transported across the membrane with bovine serum albumin (BSA). As BSA extracts all analogs from the exoplasmic monolayer of the plasma membrane, the inaccessible fraction reflects analogs that have been redistributed across the plasma membrane into cells.

The protocol presented here utilizes flow cytometry to study NBD-lipid internalization at a quantitative level in mammalian cells, exemplified on Chinese hamster ovary-K1 (CHO-K1) cells, and has been applied by us to fibroblasts (Pomorski *et al.*, 1996), lymphocytes (Fischer *et al.*, 2006), and myoblasts (Grifell-Junyent *et al.*, 2022). For lipid uptake assays optimized for fungi and plants, the reader is referred to previously published protocols (Jensen *et al.*, 2016; López-Marqués and Pomorski, 2021). The protocol includes the preparation of NBD-lipids, labelling of cells with NBD-lipids, flow cytometry measurements, and data analysis. Additionally, cells are subjected to lipid analysis via thin-layer chromatography (TLC) to assess metabolic conversion of the NBD-lipids. This protocol can be easily adapted to parasites such as *Toxoplasma* and *Leishmania* (Weingartner *et al.*, 2011; dos Santos *et al.*, 2013; Chen *et al.*, 2021), and has a broad range of applications, including: i) screening of mammalian

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cell lines for their lipid uptake profile, ii) testing the impact of gene knockouts on lipid internalization at the plasma membrane, and iii) uncovering the dynamics of lipid transport at the plasma membrane.

Things to consider before starting

A. Choice of NBD-lipid

The NBD-lipids need to fulfill three important requirements: (i) they cannot be modified at their polar head group, as this is the key structural element for substrate recognition by ATP-dependent flippases and floppases (Theorin *et al.*, 2019); (ii) they have to incorporate readily into the outer plasma membrane, to get the time zero for the uptake kinetic; and (iii) they have to be extractable by BSA. These requirements are best met by lipid analogs in which one fatty acid has been replaced by a short chain of six carbons (C6) carrying the fluorescent NBD moiety in the *sn-2* position of the glycerophospholipids, or linked by an amide bond to the ceramide backbone, as well as lyso-glycerophospholipid derivates labeled at the *sn-1* position (Figure 1).

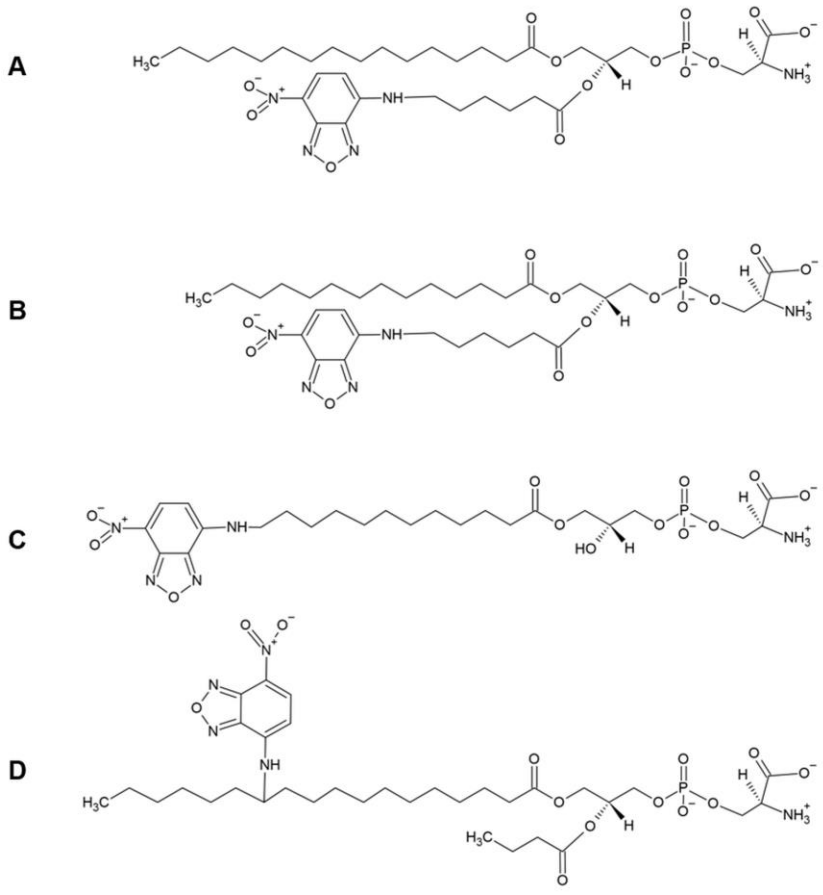

Figure 1. Chemical structure of NBD-lipids used in lipid uptake assays. Fluorescent analogs of phosphatidylserine with different acyl chain lengths and positions of the NBD moiety. **A**) 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphoserine (C16:0-C6:0 NBD-PS). In these analogs, one fatty acid has been replaced by a short chain of six carbons (C6) carrying the fluorescent NBD moiety in the *sn-2* position, whereas the *sn-1* chain is composed of sixteen carbons

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(C16). **B**) 1-myristoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphoserine (C14:0-C6:0 NBD-PS); similar to the analog in (A), but the *sn-1* chain is composed of fourteen carbons (C14). **C**) 1-NBD-dodecanoyl-2-hydroxy-sn-glycero-3-phospho-serine (NBD-lyso-PS). In these analogs, the C12 *sn-1* acyl chain carries the NBD moiety. **D**) The NBD moiety is attached to the carbon twelve of a C18 *sn-1* acyl chain, whereas the *sn-2* chain is composed of four carbons (Colleau *et al.*, 1991).

B. Metabolic conversion of the NBD-lipids

In nucleated cells, lipid internalization is not only affected by transbilayer movement and intracellular membrane trafficking, but also by lipid metabolism. Some lipid analogs are known to be actively metabolized by phospholipase activities at the cell surface. Examples are the hydrolysis of NBD-phosphatidic acid to NBD-diacylglycerol (Pagano and Longmuir, 1985), and the hydrolysis of NBD-sphingomyelin to NBD-ceramide, both occurring at the plasma membrane. The fluorescent analogs of diacylglycerol and ceramide undergo rapid spontaneous transbilayer movement, and therefore label intracellular membranes (Pagano and Sleight, 1985).

Thus, the conversion of an NBD-lipid may be followed by rapid spontaneous movement of its metabolic products and should always be critically evaluated. Another frequent modification is the hydrolysis of the NBD-lipid analogs into lyso-derivates by phospholipase A_2 activities, which results in the removal of the fatty acid attached to the *sn-2* position. The labeled C6 fatty acids liberated are released into the medium, hampering quantitative analysis of NBD-lipid internalization. To block this conversion, the assay is typically performed in the presence of phospholipase inhibitors (Pomorski *et al.*, 1996; Fischer *et al.*, 2006; Grifell-Junyent *et al.*, 2022).

Therefore, it is highly recommended to analyze the degree of NBD-lipid conversion using, for example, lipid extraction in organic solvents followed by thin layer chromatography (see under Procedure, section D).

C. Lipid internalization by endocytosis

Lipid analogs inserted into the outer plasma membrane leaflet can also be internalized by endocytosis. To suppress uptake by endocytosis, the assay is typically performed at 20°C or below.

Materials and Reagents

A. Mammalian cell culture

In this study, we used Chinese hamster ovary-K1 cells (CHO-K1; Cell number: RCB0285, Riken BRC, Japan) kindly provided by Dr. Kentaro Hanada, that were cultured in cell culture medium (see Recipes).

1. Sterile serological pipettes (*e.g.*, Serological pipettes of 5 mL, 10 mL and 25 mL; Sarstedt, catalog numbers: 86.1253.001, 86.1254.001, 86.1685.001)
2. Sterile culture vessels T-75 flasks (*e.g.*, Sarstedt, catalog number: 83.3911)

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3. Basal cell culture medium (*e.g.*, high glucose DMEM; Sigma-Aldrich, catalog number: D6546)
4. Fetal bovine serum (FBS; *e.g.*, Sigma-Aldrich, catalog number: F4135)
5. L-glutamine stock of 200 mM (*e.g.*, Sigma-Aldrich, catalog number: G7513)
6. Penicillin-streptomycin (*e.g.*, Sigma-Aldrich, catalog number: P4333)
7. Trypsin-EDTA solution (*e.g.*, Sigma-Aldrich, catalog number: T3924)
8. Hanks' Balanced Salt solution (HBSS; *e.g.*, Sigma-Aldrich, catalog number: H6648)
9. Trypan Blue Solution, 0.4% (Thermo Fisher Scientific, catalog number: 15250061)
10. Tyrode's balanced salt solution (TBSS; see Recipes)

B. Preparation of NBD-lipids
1. C16:0-C6:0 NBD-lipids purchased in chloroform including:
   NBD-PC (Avanti Polar Lipids, catalog number: 810130)
   NBD-PE (Avanti Polar Lipids, catalog number: 810153)
   NBD-PS (Avanti Polar Lipids, catalog number: 810192)
   NBD-SM (Avanti Polar Lipids, catalog number: 810218)
2. Dimethyl sulfoxide (DMSO; Carl Roth, catalog number: 4720.4)

C. Labelling of cells with NBD-lipids
1. 3-(-4-octadecyl)-benzoylacrylic acid (OBAA; Sigma-Aldrich, catalog number: SML0075)
2. Phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, catalog number: p7626)

Note: *OBAA is a potent inhibitor of phospholipase A_2 (Eintracht et al., 1998); PMSF has been reported to inhibit phospholipases, as well as some esterases (James, 1978; Estevez et al., 2012; Gadella and Harrison, 2000)*.

D. Analysis of NBD-lipid uptake and metabolism
1. PYREX® 11 mL screw cap culture tubes, 16 × 100 mm (Corning, catalog number: 9825-16), and centrifuge glasses DURAN® with conical bottoms, 12 mL (Carl Roth, catalog number: K211.1). If not using a cap (see point D2), centrifuge glasses DURAN® with round bottoms, 12 mL (Carl Roth, catalog number: C102.1) can also be used instead.
2. Tube caps with polypropylene for centrifuge glasses (Ohemen Labor, catalog number: 6702588). Using of caps is optional.
3. Polypropylene tubes of 15 mL and 50 mL capacity (*e.g.*, Falcon tubes, Sarstedt, catalog numbers: 62.554.502 and 62.547.254)
4. 1.5-mL microcentrifuge tubes (Sarstedt, catalog number: 72.690.001)
5. Tubes for flow cytometer (*e.g.*, Sarstedt, catalog number: 55.484)
6. Reusable glass media bottles with cap, 250 mL and 1 L (*e.g.*, Thermo Fisher Scientific, catalog numbers: 15456113 and 15486113)
7. Graduated pipette serological, 5 mL (Sigma-Aldrich, catalog number: BR27112)
8. Macroman™ pipette (Gilson, catalog number: F110120)

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9. Disposable glass Pasteur pipettes, 230 mm (VWR, catalog number: 612-1702)
10. Glass Pasteur pipette bulb (VWR, catalog number: 470123-222)
11. Bovine serum albumin, essentially fatty acid free (BSA; Sigma-Aldrich, catalog number: A6003)
    Note: Fatty acid-free BSA allows for efficient extraction of NBD-lipids from cellular membranes, due to the unoccupied fatty acid binding sites.
12. Propidium iodide ≥94.0% (HPLC) (PI; Sigma-Aldrich, catalog number: P4170)
13. Flow cytometer cleaning solution (*e.g.*, Sysmex, catalog number: 04-4009_R)
14. Flow cytometer sheath fluid (*e.g.*, Sysmex, catalog number: 04-4007_R)
15. Chloroform 99-99.4% ethanol-stabilized and certified for absence of phosgene and HCl (VWR, catalog number: 22711.290)
16. Ethanol absolute ≥99.8% (VWR, catalog number: 20821.321)
17. Methanol ≥99.8% (VWR, catalog number: 20847)
18. Triethylamine (Carl Roth, catalog number: X875.3)
19. Thin layer chromatography (TLC) Silica gel 60, 10 × 20 cm (Merck, Darmstadt, Germany, catalog number: 1.05626.0001)

E. Media and buffers
1. Cell culture medium (see Recipes)
2. TBSS buffer (see Recipes)
3. NBD-lipid stocks (see Recipes)
4. PMSF stock of 200 mM (see Recipes)
5. OBAA stock of 5 mM (see Recipes)
6. BSA (20% w/v) in TBSS (see Recipes)
7. PI stock of 1 mg mL^{-1} (see Recipes)
8. Alkaline running buffer (see Recipes)

Equipment

1. Analytical balance (*e.g.*, Sartorius Entris-i II, 220 g/0.1 mg, Buch Holm, catalog number: 4669128)
2. Eppendorf Research® plus pipettes P20, P200, P1000 (Eppendorf, catalog numbers: 3123000039, 3123000055, 3123000063)
3. Pipette tips 10 µL, 200 µL, 1,000 µL (Sarstedt, catalog numbers: 70.760.002, 70.3030.020, 70.3050.020)
4. Neubauer counting chamber (improved Dark lines, 0.1 mm) and cover glasses (20 mm × 26 mm × 0.4 mm)
5. Flow cabinet to work with organic solvents
6. Biological safety cabinet certified for handling of biological materials (*e.g.*, Herasafe KSP Class II Biological Safety Cabinets, Thermo Fisher Scientific)

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7. Hamilton 700 Series Syringes 25 µL, 100 µL, 1,000 µL (Hamilton Company, Nevada, USA)
8. Centrifuge with rotor for 15 mL and 50 mL polypropylene tubes (*e.g.*, Eppendorf 5810 R; Wesseling, Germany)
9. Autoclave sterilizer (*e.g.*, Systec VX-65, Systec, Linden, Germany)
10. Water distillation system
11. Incubator with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO_2 in air (*e.g.*, Binder, Tuttlingen, Germany)
12. Inverted light microscope equipped with a 10× objective (HI PLAN I 10×/0.22 PH1; Leica DMi1, Mannheim, Germany)
13. Water bath (*e.g.*, WPE45 Memmert, Schwabach, Germany) for mammalian cells and for NBD-lipid labelling (Julabo CORIO C-BT5, catalog number: 9011305)
14. Vortex mixer (*e.g.*, Vortex Genie 2 Scientific Industries Inc., catalog number: SI-0236)
15. Vacuum Pump V-100 with Interface I-100 (Buchi, catalog numbers: 11593636 and 11593655D)
16. Glass desiccator Boro 3.3 with socket in lid, 20 cm, including stopcock (BRAND GmbH, catalog number: 65238)
17. Tubing (BRAND GmbH, catalog number: 143275)
18. Developing chamber for TLC (Roth Carl, catalog number 3133.1)
19. Freezers at -20°C and -80°C
20. Refrigerator
21. Fluorescence Imager
    For this protocol, a Chemidoc MP Imaging System (Bio-Rad, Munich, Germany) with a 530/28 filter and light Blue Epi illumination was used.
22. Flow cytometer
    For this protocol, a CyFlow® SL flow cytometer (Sysmex Partec, Münster, Germany) was used, equipped with a solid-state laser of 488 nm.
23. Computer with monitor (*e.g.*, DELL U2415)

Software

1. FloMax® software (Sysmex Partec)
2. FlowJo™ v10.0.7 Software (FlowJo)
3. Image Lab™ software (Bio-Rad)

Procedure

When utilizing cell lines that grow in suspension, skip Steps A1–A5 and start at Step A6.

A. Preparation of mammalian cells

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1. Grow adherent cells in sterile culture vessels (T-75 flask) in appropriate cell culture medium in a tissue culture incubator (37°C, 5% CO_2, 95% humidity) until they reach a confluency of 70–80%.
   Note: Given that for each time point and NBD-lipid, at least ~10^4 cells are required, a total of 3 × 10^6 cells were collected per lipid tested, which corresponded to three T-75 flask of CHO-K1 cells at a confluency of 70–80%. For other cell lines, growth conditions and confluency degrees might require adjustment.
2. Remove media and wash cells twice with 5 mL of HBSS (Ca^{2+} and Mg^{2+} free, pre-warmed at 37°C).
3. Add 1.5 mL of trypsin-EDTA solution (pre-warmed at 37°C) and incubate the T-75 flasks in a tissue culture incubator (37°C, 5% CO_2, 95% humidity).
4. After 5 min, check under the microscope if the cells have detached. If the cell line allows it, tap the flask in the side to help the detachment of the cells from the bottom.
   Note: Cells in suspension will show a round-shaped morphology.
5. Stop trypsinization by adding 7.5 mL of appropriate cell culture medium (pre-warmed at 37°C).
   Note: For cells grown in suspension, Steps A1–A5 are omitted.
6. Transfer the cell suspension into a 50 mL Falcon and set aside 50 µL of this in a 1.5-mL microcentrifuge tube for cell counting, *e.g.*, using the hemocytometer (see below).
7. Centrifuge cells in the 50 mL Falcon tube at 500 × *g* and room temperature for 10 min and discard the supernatant.
8. Add 10 mL of TBSS buffer (see Recipes) at room temperature to the cell pellet, and resuspend completely by pipetting up and down.
   Note: This additional washing step ensures the removal of phenol red, a pH indicator present in DMEM media that interferes with the NBD-lipid fluorescence quantification.
9. Centrifuge the cells in the 50 mL Falcon tube at 500 × *g* and room temperature for 10 min and discard the supernatant.
10. Resuspend the cells in TBSS buffer (see Recipes) to a final concentration of ~10^6 cells mL^{-1}.
11. To block the conversion of NBD-lipids by cellular phospholipases, add PMSF and OBAA to a final concentration of 1 mM and 5 µM, respectively, to the cell suspension.
12. Gently mix and incubate at 20°C in a water bath placed in a cold room for 10 min. The assay is typically performed at 20°C or below to suppress endocytosis.

B. Cell counting

1. Prepare the hemocytometer by cleaning the chambers and coverslip with isopropanol. Dry the hemocytometer by using lint-free tissue. Place the glass cover slip over the counting chambers.
2. Add 50 µL of 0.4% trypan blue stock solution to 50 µL of cell suspension (Step A6) to obtain a 1:1 dilution.
3. Load the hemocytometer with 10 µL of cell suspension and examine immediately under an inverted phase contrast microscope at low magnification.

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4. Count the number of viable (seen as bright cells) and non-viable cells (stained blue) in the large outer quadrants.
5. Calculate the percentage of viable cells: % viable cells = [1.00 – (Number of blue cells ÷ Number of total cells)] × 100. Cell viability should be at least 95%.
6. Calculate the cell concentration, based on the premise that each square accounts for a volume of 10^{-4} mL of cell suspension.
7. To obtain the total number of viable cells per ml of aliquot, multiply the total number of viable cells by 2 (the dilution factor for trypan blue) and the correction factor of 10^4 (volume of each square).

C. NBD-lipid uptake assay

Note: In order to prevent nonspecific binding of the NBD-lipids, all steps must be performed in glass tubes.

1. Prior to the start of the assay, prepare round bottom glass tubes with NBD-lipid stocks in DMSO (see Recipes) and 1.5-mL microcentrifuge tubes on ice: one empty and one with 30 µL of 20% BSA (see Recipes) for each time point.
   Note: We routinely use a final BSA concentration of 4.6% (w/v) for NBD-lipid extraction. However, the amount of BSA required for extraction, as well as the incubation time, may vary depending on cell type and lipid analog used in the assay (Fellmann et al., 2000). To determine the optimal conditions, label the cells at 4°C and measure the cell-associated fluorescence after different time of contact of the cell suspension with BSA.
2. To start cell labeling, add 3 mL of the cell suspension (from Step A12) into the glass tubes containing the NBD-lipid stocks, and gently vortex for 2 s (Figure 2).
3. Incubate cells with NBD-lipids for the desired time periods, *e.g.*, 0, 5, 10, 20, 30, 45 and 60 min. Note that for t=0 min, samples need to be collected immediately after addition of cells to the lipid suspensions. When analysing uptake of several NBD-lipids in parallel, start cell additions spaced by 1 min intervals.
4. At each time point, gently vortex the cell suspensions for 2 s using low vortex power settings to avoid pelleting of the cells, and take two aliquots of 100 µL. Transfer one aliquot to a pre-cooled empty 1.5-mL microcentrifuge tube, and the other aliquot to a precooled 1.5-mL microcentrifuge tube containing 30 µL of 20% BSA (see Figure 2).
   Note: At this point, additional samples can be taken for analyzing metabolic conversion of the fluorescent lipid analogs using lipid extraction and thin layer chromatography analysis (see section D).
5. Keep the samples on ice for a maximum of one hour after collecting the last sample, and analyse them via flow cytometry.
6. Turn on the CyFlow® SL flow cytometer, start the computer, and open the FlowMax software.
7. Click on the panel “Instrument Settings” and manually enter the parameters (Table 1).
   Note: Save the employed parameters on the instrument to reload in future runs.

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8. Open the gating menu, select “Polygon” and click on “New”. Generate a gate in the plot, by default termed R1, with the parameters forward scatter (FSC) in the x-axis and side scatter in the y-axis (Dawaliby *et al.*, 2016), covering most of the surface in the center of the plot.
   Note: Gates can be saved and loaded in future runs.
9. Click on the option “Setup” to open the Setup window. Select as the maximal count number for R1 10,000 cells. Click “OK”.
   Note: We typically count cells until reaching 10,000 in gate R1 (corresponding to population P1).
10. To rinse the machine before use, add 1 mL of TBSS into a flow cytometer tube and run for about 1 min at a speed between 4–8 µL s^{-1}.
    Note: Make sure there are very little to no counts and thus, no contamination or residual cells in the flow cytometer. In case of contaminants, rinse for longer times with TBSS and, if still persistent, wash with the cleaning solution, and then again with TBSS.
11. Set the flow speed at 3–4 µL s^{-1} for counting on average 300 cells s^{-1}. Speed can be adjusted if necessary.
12. Transfer the cell sample into a flow cytometer tube, followed by addition of 1 µL of 1 mg mL^{-1} PI and 1 mL of TBSS. Shortly vortex and place the sample tube in the sample holder. PI labelling allows excluding dead cells from the analysis, which readily absorb NBD-lipids because of the disruption of their plasma membranes. Upon addition of a new cell sample, a message appears to save the data just collected. In the equipment used, samples are automatically saved in the .fcs format (check “Data analysis” on how to proceed for samples analysis).

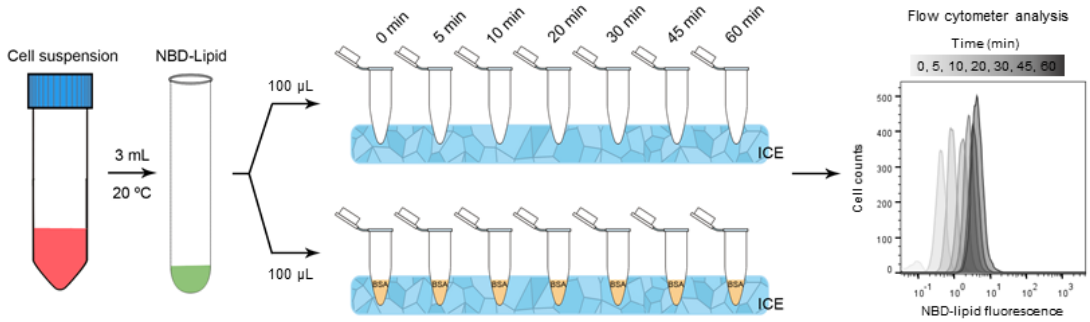

Figure 2. Schematic illustration of the NBD-lipid uptake assay. For labeling, cells (10^6 cells mL^{-1}) pre-incubated with phospholipase inhibitors at 20°C are transferred to glass tubes containing the DMSO-dissolved NBD-lipid, and then incubated for different time periods. At each time point, two aliquots of 100 µL are taken. One aliquot is transferred to a pre-cooled empty 1.5-mL microcentrifuge tube, and the other aliquot to a precooled 1.5-mL microcentrifuge tube containing 30 µL of 20% BSA. Samples are subsequently analysed by flow cytometry. Details on the individual steps are described in the text.

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Table 1. Parameters employed for flow cytometry analysis of the mammalian cell line CHO-K1 labelled with NBD-lipids.

Settings employed for each parameter, including gain, scale (Log), lower limit (L-L) and upper limit (U-L). FSC, forward scatter; SSC, side scatter; FL1, channel for NBD fluorescence; FL3, channel for PI fluorescence. The flow cytometer used is equipped with a blue solid state laser (488 nm, 20 mW). FL1 and FL3 fluorescence are recorded with a band pass filter IBP 527/30 and a long pass filter RG 630 nm, respectively. Note that these values might require adjustment depending on the cell line and/or flow cytometer employed.

Parameter	Gain	Log	L-L	U-L
FSC	170	lin	110	999.9
SSC	190	lin	10	999.9
FL1	220	Log4	10	999.9
FL3	415	Log4	10	735.9

D. Analysis of fluorescent lipid metabolism

Lipid extraction can be performed based on the modified (Bligh and Dyer, 1959) protocol. The method is based on the partitioning of lipids in a biphasic mixture of chloroform and methanol. Methanol disrupts hydrogen bonds between lipids and proteins following addition of an organic solvent such as chloroform. Except centrifugation, all steps described are performed in a flow cabinet to avoid direct exposure to organic solvents.

1. Transfer aliquots of the cell suspension at the given time points to 12 mL glass tubes. We typically analyse the metabolism after 60 min, by taking volumes corresponding to ~4 nmol NBD-lipid (150 µL of cells labelled with NBD-PE; 300 µL of cells labelled with NBD-PC; 600 µL of cells labelled with NBD-PS or NBD-SM).
2. Add up to 2 mL of ddH_2O considering the volume already present in each glass tube.
3. Add 2.2 mL of methanol followed by 2 mL of chloroform using glass graduated pipettes and vortex carefully for 3–5 s to achieve a single phase.
4. Centrifuge the tubes at 500 × *g* for 5 min to obtain two separated phases.
5. Transfer the lower phase (chloroform phase containing lipids) into a conical bottom glass tube using a glass Pasteur pipette.
6. For a second round of extraction, add 1 mL of chloroform to the original tubes, vortex shortly and centrifuge again at 500 × *g* for 5 min.
7. Transfer the lower phase into the glass tubes from Step D5.
8. Prepare four conical bottom glasses and add 4 nmol of NBD-PC, NBD-PE, NBD-PS and NBD-SM resuspended in chloroform:methanol (1:1, v/v) from the same lipid stocks used to label the cells. These samples will serve as standards to compare to the extracted lipid samples.
9. Dry both the lipid extracts and the standard samples under vacuum at 250 mbar overnight or under a gentle stream of nitrogen gas for 30 min to 1 h.

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10. On a TLC silica plate, mark 1 cm lines as loading spots with a pencil. These should be 1.5 cm apart from the bottom edge and 1 cm apart from the side edges and from each other. Draw a total of eight lines, four for the NBD-lipid standards and four for the extracted NBD-lipid samples (Figure 3A). Samples information can be written by pencil under the loading spots. Do not use excessive force when writing on a TLC plate as this will remove the silica coating.
11. Carefully pour the running buffer (see Recipes) into the developing chamber until filled to ~0.5 cm of heigh. Close with the lid to allow air saturation for ~20 min.
12. Dissolve the extracted dried lipids (~4 nmol) and the 4 nmol NBD-lipid standards in a volume of ~5 µL of chloroform/methanol (1:1, v/v).
13. Apply the dissolved NBD-lipids onto the TLC silica plate using a glass Pasteur pipette, by placing it carefully on top of the 1 cm loading spot to avoid peeling off the silica and ensuring even distribution. Let the organic solvent evaporate before adding more sample on top on the loading spot.
14. Place the TLC plate as evenly as possible in the chamber with a slight tilt. Close the lid and run for 20–25 min, or until the running front has reached a distance of 1 cm from the top edge.
15. Remove the TLC plate from the chamber, mark the running front with a pencil, and let it dry for 15–30 min.
16. Image NBD-lipid fluorescence in the Chemidoc Imaging System, using the Image Lab™ software and emission filter 530/28 nm under Blue light Epi illumination (Figure 5C).
    Note: Place the TLC plate on top of a transparent plastic bag to avoid scratching the Chemidoc tray surface.
17. Optional: Band intensities can be quantified using the Image Lab™ software, *e.g.*, to assess the percentage of metabolic conversion of lipids. Low hydrolysis (<10%) does not affect the NBD-lipid internalization kinetics. However, for higher hydrolysis (>10%) it is crucial to determine whether the converison occurs intracellularly or on the cell surface, to interpret the results.

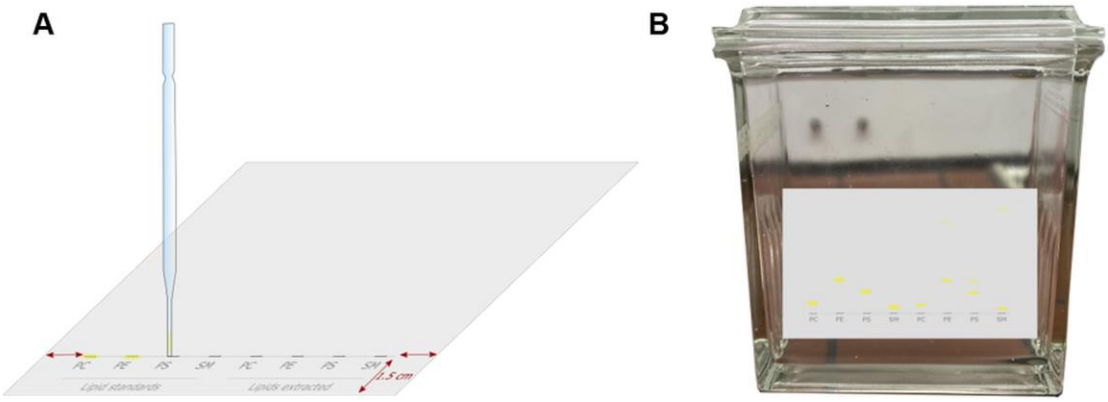

Figure 3. Preparation of the TLC. A) Marking of the loading spots on the silica plate with a pencil and loading of the NBD-lipids with a glass Pasteur pipette (for details see text). The lanes should not be placed too close to the edge: keep a distance of 1.5 cm as indicated by red arrows. **B**) Silica plate placed into the TLC chamber containing the alkaline running buffer and covered

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with the glass lid. The plate is developed until the solvent is about half a centimeter below the top of the plate.

Data analysis

1. Export data in original format (.fcs) from the flow cytometer and import into the FlowJo sofware.
2. Double click on top of the first file. A plot will open and cells can be visualized as dots. Select FCS and SSC for the x and y-axis respectively, and selecte the logarithmic scale. Adjust the scale range.
3. Generate a gate around the cell population and label it as P1, referring to CHO-K1 cells in this case (Figure 4A). Double click on top of the selected population, another panel plot will open.
4. Select FL3 (PI fluorescence) for the x-axis and keep SSC for the y-axis in the new plot. Generate a gate around the cell population with low PI-fluorescence, which includes only alive cells, and label as P2 (Figure 4B).
5. Double click on top of the P2 population. A new plot will open. Select FL1 (NBD-lipid fluorescence) for the x-axis and SSC (side scatter) for the y-axis (Figure 4C).
6. In the FlowJo interface, the populations P1 and P2 created appear below the .fcs file. Right-click on each and select “Copy analysis to group” to apply gates to all the other files.
7. Check that the generated gates fit for all samples, and move to fit if necessary.
8. In the FlowJo interface, click on Workplace and then click in Add Statistics. Select “Geometrical Mean”, population P2, and parameter FL1.
9. Save analysed samples as Worspace (WSP) files and as Excel (xls) files.
10. Calculate the percentage of NBD-lipid that cells have uptaken at each timepoint (Figure 5A, B). The geometrical mean of each timepoint of the sample without BSA addition is considered as the 100% NBD-fluorescence. The geometrical mean value of the sample with BSA at a given time point is the relative percentage compared to the value assigned to the 100% (from the sample without BSA; Eq. 1). The new calculated percentage is referred to as non-extractable NBD-lipid in step 11 and Eq. 1 or as NBD-lipid uptake in Figure 5B and elsewhere. Plot the resulting percentages of internalized NBD-lipid at each time point (Figures 5A and 5B).

$$\text{Non-extractable NBD-lipid } (\%) = \frac{\text{geometrical mean value of sample with BSA}}{\text{geometrical mean value of sample without BSA}} \times 100 \quad \text{Eq. 1}$$

11. Fit data according to the equation for a single-exponential curve (*e.g.*, using Microsoft Excel and the add-in programm Solver):

$$y = a + b * (1 - e^{c*t}) \quad \text{Eq. 2}$$

where

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y is the fitted value for NBD-lipid fluorescence in percentage (%) at each time point,

t is the time after NBD-lipid addition,

a is the percentage of non-extractable NBD-lipid at t=0,

b is the percentage of non-extractable NBD-lipid at steady state,

c is the rate coefficient.

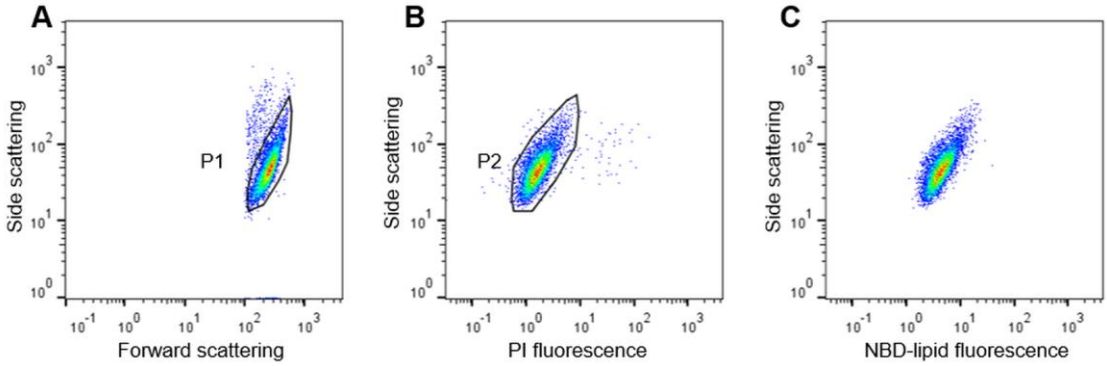

Figure 4. Gating cell populations using the FlowJo software. Cells were labelled with NBD-lipids and collected at a given time point. This data illustrates the gating procedure corresponding to cells labelled for 60 min and treated with BSA. **A**) Cells were visualized in a logarithmic scale plot of side- and forward-scattering. A population P1 was defined as corresponding to mammalian cells and excluding other possible particles (*e.g.*, cell debris). **B**) The P1 population was visualized in a new panel plotting side scattering and red propidium iodide (PI; dos Santos *et al.*, 2013)-fluorescence. A population P2 was defined by selecting alive cells and excluding dead cells, labelled with PI. **C**) The P2 population was subsequently plotted in a side-scatter and green NBD-fluorescent panel, thus showing alive cells that internalized NBD-lipids.

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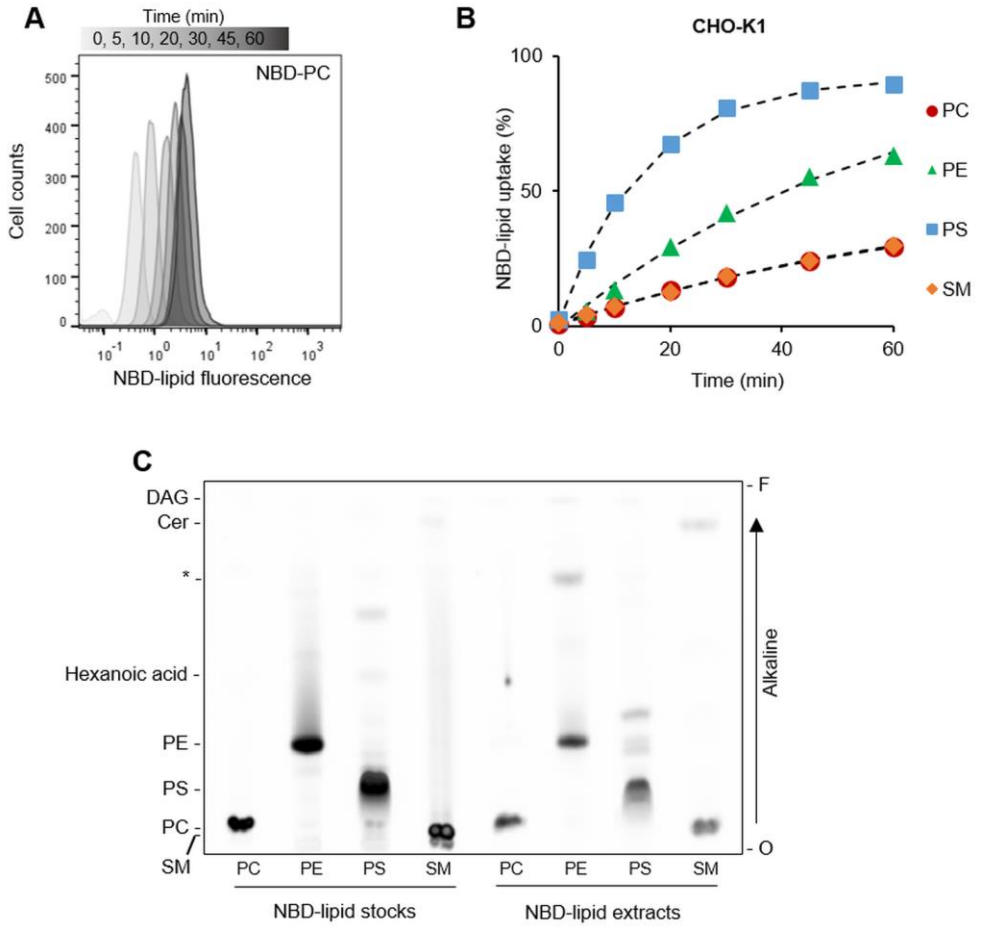

Figure 5. Exemplary time lapse of NBD-lipid uptake in CHO-K1 cells and lipid metabolism. Cells incubated with the indicated NBD-lipids at 20°C were analysed by flow cytometry after back-extraction to BSA. **A**) Histogram of NBD-PC fluorescence intensity in CHO-K1 cells after BSA incubation measured at the indicated timepoints. Note that a corresponding histogram for cells without BSA treatment will show a constantly high cell-associated fluorescence over time, provided that there is no metabolic modification, *e.g.*, hydrolysis of the NBD-lipid analogs into lyso-derivates and free NBD-fatty acid. **B**) Time course of NBD-lipids internalization in CHO-K1 cells, shown as percentage of fluorescence intensity relative to fluorescence from cells treated in the absence of BSA. Plotted lines represent the best fit to a single-exponential curve. **C**) Thin layer chromatogram of NBD-lipids extracted from CHO-K1 cells after 60 min incubation and after BSA treatment. Lipid samples were resolved onto a silica plate along with standards and imaged for fluorescence. Diacylglycerol (DAG) ceramide (Cer) and NBD-X [6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic acid] (hexanoic acid), products of NBD-lipids metabolic conversion are also indicated. The asterisk (*) indicates either unidentified contaminants or lipid species derived from NBD-PE. The arrow on the right shows the running direction of the alkaline buffer. The running origin (O) and running front (F) are marked. NBD, nitrobenzoxadiazol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin.

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Recipes

Buffers were prepared using double distilled water (ddH_2O), which was obtained using an in-house water distillation system. Alternatively, all buffers are prepared using ultrapure water with purification sensitivity of 18 MΩ cm^{-1} at 25°C.

1. Cell culture medium
   Open a 500 mL flask of high-glucose DMEM medium
   Add 50 mL of fetal bovine serum
   Add 5 mL of L-glutamine
   Add 5 mL of penicillin-streptomycin
   Prepare in sterile cabinet; store at 4°C
2. TBSS buffer
   136 mM NaCl
   2.6 mM KCl
   1.8 mM $CaCl_2$
   1 mM $MgCl_2$
   0.36 mM NaH_2PO_4
   5.56 mM glucose
   5 mM HEPES
   Adjust pH to 7.4 with NaOH. Complete volume to 1 L. Sterilize by filtering using a 0.22 µm filter. Store at 4°C for up to several months.
3. NBD-lipid stocks
   All steps must be performed in glass tubes in order to prevent nonspecific binding of lipids.
   Note: Chloroform is a chemical hazard. Do not breathe gas/fumes/vapor/spray. Wear suitable protective clothing. Work in a fume hood.
   a. Use a glass syringe to transfer the desired amount of NBD-lipid into a disposable 12 mm-diameter glass tube. Typically, we label 3 mL of cell suspension with 80 nmol of NBD-PE, 40 nmol of NBD-PC, and 20 nmol of NBD-PS or NBD-SM.
      Note: These amounts have been optimized to allow for using the same flow cytometry settings when analysing the labeled cells.
   b. Dry the lipids under a 250 mbar vacuum overnight or under a gentle stream of nitrogen gas for 30 min to 1 h, so that a dried lipid film is formed at the bottom of the tube.
   c. Resuspend the NBD-lipids in 20 µL of DMSO shortly before use. Add the DMSO in a circular fashion, by placing the piepette tip onto the walls of the glass tube and on top of the dried lipid film. Slowly pipette up and down repeating the circular movement until all the lipid film is resuspended.
   Note: DMSO lipid suspensions are prone to precipitation owing their hygroscopic nature and should be freshly prepared. DMSO is a chemical hazard. Wear suitable protective clothing.
4. PMSF stock of 200 mM

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Weight 34.838 mg with protective gear to avoid inhalation or contact with skin.
Dissolve in 1 mL ethanol.
Store at -20°C.
Note: PMSF is a chemical hazard, specially in its solid state, highly toxic if swalled, and causing severe skin burns and eye damage upon contact. Wear suitable protective clothing and work in a fume hood when preparing the stock solution. Handle the stock solution carefully.

5. OBAA stock of 5 mM
   Weight 2.14 mg
   Add 1 mL of absolut ethanol
   Store at -80°C
   Note: Wear suitable protective clothing.
6. BSA (essentail fatty acid-free, 20% w/v) in TBSS
   Weight 200 mg BSA in a 15 mL Falcon tube
   Add 1 mL of TBSS
   Incubate at 37°C in a water bath until partially dissolved.
   Store in the fridge until next day and use within one week; do not freeze the solution.
7. PI stock of 1 mg mL^{-1}
   Weight 1 mg of PI
   Add 1 mL of ddH_2O
   Store at -20°C
   Note: PI is a suspected carcinogen and should be handled with care. The dye must be disposed of safely and in accordance with applicable local regulations. Wear suitable protective clothing.
8. Alkaline running buffer
   Mix the following volumes in 30:35:35:7 v/v/v/v (mL) chloroform:ethanol:triethylamine: ddH_2O in a blue-cap glass.
   Shake vigourously. Prepare on the same day of the experiment.
   Note: For the size of the TLC chamber (W × D × H: 235 × 116 × 220 mm) described in this protocol, the alkaline running buffer was prepared by mixing 15:17.5:17.5:3.5 v/v/v/v (mL) chloroform:ethanol:triethylamine: ddH_2O. The organic solvents are chemical hazard. Do not breathe gas/fumes/vapor/spray. Wear suitable protective clothing. Work in a fume hood.

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Competing interests

The authors declare that no competing interests exist.

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